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(57) Abstract			
Modified TCF in which amino-acid residue(s) of the wild-type TCF responsible for glycosylation is (are) substituted or deleted so that at least one N-linked oligosaccharide chain is removed. The modified TCF have longer biological half-lives without loss of its biological activities. The modified TCFs are therapeutically important as agents for liver diseases or as anti-cancer drugs.			

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DESCRIPTION

MODIFIED TCF

FIELD OF THE INVENTION

This invention is related to genetically engineered modified TCFs which contain different number of N-linked oligosaccharide chains compared with wild type TCF, and have new amino-acid sequences. These modified TCFs obtained in this invention have longer serum half-lives, have growth stimulating activities for hepatocytes and cytotoxic activities against tumor cells. Therefore, the modified TCFs are useful as therapeutic agents against liver diseases and as anti-cancer drugs.

BACKGROUND OF THE INVENTION

TCF-II, tumor cytotoxic factor derived from human fibroblast, is a novel anti-tumor protein which is different from any other proteins so far reported. The inventors succeeded in cDNA cloning of this protein, deduced its entire amino-acid sequence and confirmed the usefulness. This novel anti-tumor protein and its cDNA were disclosed in W090/10651. They were designated as TCF-II. In this invention, the glycoprotein which has the amino-acid sequence disclosed in W090/10651 is called TCF. TCF is a substance which has been called TCF-II.

TCF has both strong cytotoxic activity against tumor cells and growth stimulating activity for normal cells. And it has been confirmed that TCF is a member of a family including HGF, a growth factor for hepatocytes. The molecular weight of TCF is 78000 ± 2000 daltons and/or 74000 ± 2000 daltons on SDS polyacrylamide gel electrophoresis. Under reducing conditions, it showed a polypeptide band called A chain with a molecular mass of 52000 ± 2000

daltons and two polypeptide bands called B chain and/or C chain with molecular masses of 30000 ± 2000 daltons and/or 26000 ± 2000 daltons, respectively.

Because TCF is a growth factor for hepatocytes, application to liver regeneration after hepatectomy has been examined. Since biological half-life of TCF is very short, attempts have been made to obtain more effective modified TCFs with prolonged biological half-lives.

Relationship between oligosaccharide chains and serum half-lives has been investigated in some glycoproteins including erythropoietin. The investigations have demonstrated that glycoproteins with slightly different structure in oligosaccharide chains and with different biological activities can be synthesized from same gene. It was known that glycosylated erythropoietin is different in the biological activities from non-glycosylated one. However, about the relationship between oligosaccharide chains and biological activities of TCF little has been known.

DISCLOSURE OF THE INVENTION

The present inventors took notice of the usefulness of TCF, and investigated application of TCF to the treatment of tumors or liver diseases and utilization as diagnostic markers of diseases. TCF has a very short half-life of approximately 2 minutes. To obtain modified proteins with prolonged biological half-lives, the inventors constructed several genetically engineered TCFs with mutations in the polypeptide moiety and analyzed them. However, most of the modified proteins appeared to lose the biological activities. Then, to prolong the biological half-life without loss of the biological activity, the inventors paid attention to the four N-linked oligosaccharide chains attached to TCF and attempted to construct the new modified TCFs which have a deletion of one or more oligosaccharide chains.

The theme of this invention is to present the new modified TCFs which have different amino-acid sequences compared to the wild-type TCF, and decreased number of N-linked oligosaccharide chains and longer biological half-lives than the wild-type TCF. These modified TCFs can be obtained by altering the nucleotide sequence of TCF cDNA encoding the amino-acid residues responsible for N-glycosylation and by expressing the genetically mutagenized TCF cDNAs. Modified TCFs presented by the invention have one or more deletion(s) of the four N-linked oligosaccharide chains present in the wild-type TCF. These are the first modified TCFs with prolonged biological half-lives without any loss of the biological activities, obtained by altering the amino-acid residues of the wild-type TCF.

Fig. 1 shows the entire amino-acid sequence of the wild-type TCF deduced from its cDNA sequence (Seq. Id No. 1). Amino-acid residues responsible for N-glycosylation are underlined. It is speculated that the signal sequence is removed and the glutamine residue at position 32nd results in the N-terminal amino acid of TCF. Four N-linked oligosaccharide chains bind to asparagine residues at positions 258th, 366th, 530th, and 617th relative to the N-terminal amino acid. These asparagine residues correspond to those at positions 289th, 397th, 561st, and 648th in Fig.1.

Fig. 2 shows cDNA sequence encoding the amino-acid sequence. The coding region starts at the ATG codon marked with a circle in the figure.

Amino-acid residues responsible for N-glycosylation are Asn-X-Thr or Asn-X-Ser (amino-acid residues are indicated by the three-letter code and the X represents any amino-acid residues). Modified TCF lacking specific oligosaccharide chain(s) can be obtained by expressing the nucleotide sequence in which the codons for Asn, Ser or Thr are deleted or substituted with those for one of the other amino acids, in eukaryotic cells, preferably mammalian cells. Modified TCF which lacks specific oligosaccharide chains can be obtained

by replacing the codons for Asn with those for other amino acids such as Gln, a substitution with little effects on the conformation of TCF. Gln is the most preferable amino acid which substitutes for Asn. Asp, Glu, His, Ser or Thr are also acceptable. Modified TCF lacking specific oligosaccharide chain(s) can be obtained by replacing the codons for Ser with those for other amino acids such as Ala, a substitution with little effects on the conformation of TCF. Ala is the most preferable amino acid which substitute for Ser. Pro, Gly or Asn are also acceptable. Modified TCF lacking specific oligosaccharide chain(s) can be obtained by deleting or replacing the codons for Thr to other amino acids. Ala is the most preferable amino acid which substitutes for Thr. Val, His or Asn are also acceptable.

N-glycosylation sites are indicated in a schematic structure of TCF in Fig. 3. The N-glycosylation sites are designated as numbers 1, 2, 3 and 4 from N-terminal. These numbers specify the positions of the N-glycosylation sites.

To alter the amino-acid sequences responsible for N-glycosylation into those for non-glycosylation, the cDNA can be site-specifically mutagenized by PCR(polymerase chain reaction). The reactions are performed by cDNA for the wild-type TCF as the template and synthetic oligonucleotides as the primers. The sequences of primers are designed to delete or substitute the DNA sequences as described above. Other methods for mutagenesis are also applicable.

The cell lines producing the modified TCFs can be established by transforming host cells with expression vectors containing these mutant cDNAs. The modified TCFs can be recovered from the cultured broth of the transformed cell lines.

For example, to substitute Asn at position 289th (Fig. 1), binding site for oligosaccharide chain 1, cDNA was mutagenized by in vitro mutagenesis or mutagenesis by PCR. These mutagenesis reactions are performed by cDNA coding the wild-type TCF disclosed in WO 90/10651 as the template and synthetic

oligonucleotide TCF-1R 5'-TCAGTGCCTGCATAGTAT-3' as the primer. An expression vector containing the mutagenized cDNA can be transfected into eukaryotic cell lines including mammalian cell lines. The modified TCFs can be recovered from the cultured supernatants of the transfected cells.

Any types of host-vector systems for eukaryotic cells are acceptable for expression of the modified TCFs. The most preferable is a combination of cytomegalovirus promoter and Namalwa cells, which was disclosed in WO 92/1053. Commonly used systems including a gene amplification system using a combination of SV 40 promoter, DHFR gene and CHO cell line or an expression system using a combination of the replication origin of bovine papiloma virus and mouse C127 cell line can be enumerated.

Any commonly used methods for purification of biologically active proteins, can be used for purification of the modified TCFs, for example, precipitation by organic solvent, salting out, gel exclusion chromatography, affinity chromatography using monoclonal antibody or electrophoresis. Monoclonal antibodies against the wild-type TCF disclosed in Japanese Patent application number, 3-177236 can be used for affinity chromatography of modified TCF.

The modified TCFs can be stored lyophilized or frozen.

Fifteen modified TCFs can be obtained by combinations of the removal of the oligosaccharide chains. The modified TCFs are identified by numbers of removed oligosaccharide chains. For example, the modified TCF which lacks oligosaccharide chain 1 is named TCF-1, the modified TCF which lacks all four oligosaccharide chains is named TCF-1234, and the modified TCF which lacks oligosaccharide chains 2 and 3 is named TCF-23. Figure 4 schematically represents the N-linked oligosaccharides bound to the modified TCF. All the modified TCFs have different molecular masses according to the removal of the oligosaccharide chains.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the entire amino-acid sequence of the wild-type TCF deduced from its cDNA sequence. Amino-acid residues responsible for binding of N-linked oligosaccharide chains are underlined.

Figure 2 shows the cDNA sequence for the wild-type TCF. Codons to be substituted in the present invention are underlined. The coding sequence starts at the ATG codon marked with a circle.

Figure 3 schematically represents the primary structure of the wild-type TCF. Circles represent the N-glycosylation sites and the arrow shows the cleavage site between A chain and B chain.

Figure 4 schematically represents the oligosaccharide chains attached to TCF and the modified TCF. The horizontal lines represent the polypeptides and the vertical bars represents the oligosaccharide chains.

Figure 5 shows the construction of the plasmids containing cDNAs for the modified TCF.

Figure 6 shows the construction of the expression vectors for TCF and the modified TCF.

Figure 7 shows the analysis of the representative modified TCF by SDS polyacrylamide gel electrophoresis.

Figure 8 shows the stimulating activities of the modified TCF for growth of hepatocytes. Each figure shows the activity of the modified TCF described below.

- (1) TCF, TCF-1, TCF-2, TCF-3 and TCF-4
- (2) TCF-12, TCF-13, TCF-14, TCF-23, TCF-24 and TCF-34
- (3) TCF-123, TCF-124, TCF-1234, TCF-134 and TCF-234

Figure 9 shows the tumor cytotoxic activities of the modified TCF.

Figure 10 shows the plasma concentrations of the modified TCF in the

rats into which said modified TCFs were intravenously injected.

The novel modified TCFs are obtained by operating the present inventions. The modified TCFs obtained by the present invention can be prepared by expressing the cDNAs in which the codons for the amino-acid residues responsible for N-glycosylation are substituted with those for other amino acids or are deleted. The modified TCFs have longer biological half-lives due to the control of the numbers and the binding sites of the N-linked oligosaccharide chains.

BEST MODE FOR PRACTICE OF THE INVENTION

In the examples herein, the present invention is described in more detail.

Example 1

Preparation of the modified TCFs.

DNAs were manipulated essentially as described in Molecular Cloning, A Laboratory Manual, Second Edition, J. Sambrook, E.F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, New York, 1989 to prepare modified TCFs as described below.

① Cloning of TCF cDNA.

Site-specific mutations were introduced into TCF cDNA as described below by a 6.3-kb TCF expression plasmid, pcDTCF II, prepared by the method disclosed in W092/01053. The E. coli strain containing pcDTCF II has been deposited to National Institute of Bioscience and Human-Technology as FERM BP-3479.

② Methods for introducing site-specific mutations.

1) Method 1

i) Cloning of TCF cDNA into M13mpl8.

The plasmid pcDTCFII (6.3 kb) was prepared by introducing the entire coding region of human TCF cDNA into plasmid pcDNA I (Invitrogen Co.) digested with restriction enzymes BamHI and SphI (Takara Co.). (All the restriction enzymes and the modifying enzymes were purchased from Takara Co.) pcDTCFII was digested with BamHI and SphI for 1 hour at 37°C, precipitated with ethanol, and electrophoresed on 1% agarose gel. The 2.3 kb of TCF cDNA fragment was purified from the gel by Gene Clean (Bio101 Co.).

On the other hand, replicative-form of phage DNA, M13mpl8 (Takara Co.) was digested with BamHI and SphI, precipitated with ethanol, and dissolved in water to make the vector DNA solution. The vector DNA solution and TCF cDNA were mixed and ligated by the DNA Ligation Kit (Takara Co.). A part of the ligation mixture was used for transformation of *E. coli*, DH5 α . The transformants were mixed with *E. coli*, NM522, an indicator strain, (Invitrogen Co.) and poured onto the LB soft agar plates containing 1% of bacto-tryptone, 0.5% of bacto-yeast extract, 1% of sodium chloride, 1% of IPTG (isopropyl- β -D-thiogalactoside, Takara Co.), and 1% of Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactoside, Takara Co.) in order to make plaques. The double-stranded phage DNA was prepared from a clear plaque and the single-stranded DNA was prepared and used as a template for introducing site-specific mutations.

ii) Introduction of site-specific mutation.

The codon for the 289th amino acid, Asn, in a consensus amino acid sequence for N-glycosylation, Asn-X-Thr, was replaced with a codon for Gln that does not make the consensus amino-acid sequence for N-glycosylation.

The definition of the number of amino acid is based on Met at the N-

terminal of the wild-type TCF as the first amino acid. The oligonucleotide TCF-1R, 5'-TCAGTGTCTGCATAGTAT-3', was synthesized by a DNA synthesizer (Applied Biosystems Co.). All the oligonucleotides were synthesized by the synthesizer unless otherwise noted.

Introduction of site-specific mutation was carried out by the oligonucleotide-directed *in vitro* mutagenesis system (Amersham Co.) according to the manufacture's instruction. E. coli, NM522 was transformed with the reaction mixture and screened with TCF-1 primer by the plaque hybridization technique.

The single-stranded DNA was prepared from a positive plaque and the sequence was determined by the dideoxy chain termination method described by Sanger et al. to confirm that the codon, AAT, for Asn at 289th, was replaced with the codon, CAG, for Gln. The 1.4-kb PstI-HindIII DNA fragment was prepared from the double-stranded DNA from the positive clone, ligated to PstI-HindIII-digested pBluescript SK+ (Stratagene Co.) to produce plasmid, pSKTCFPH-1.

2) Method 2

Introduction of site-specific mutations in the codons for the 399th, 563rd, and 650th amino acids were carried out essentially as described by R.Higuchi (R.Higuchi, PCR protocols, p.177-183, Academic Press, 1990) using polymerase chain reaction (PCR). A codon for Ser in a consensus amino acid sequence for N-glycosylation, Asn-X-Ser, was replaced with a codon for Ala in each mutagenesis.

To mutagenize the codon for Ser at 399th, 25 cycles of PCR were carried out by a mutant primer, TCF-2R 5'-CCAGATCTTGTGTTGAGCTAAGTTGCCC-3', a wild type primer, TCF701F 5'-GCTGGGATCATCAGACACCAC-3', AmpliTaq polymerase (Takara Co.), and 4 ng of the plasmid pcDTCF II as a template. The PCR products were purified by centricon 100 (Amicon Co.) to remove primers, digested with restriction

enzymes, BglII and EcoRI (Takara Co.), and ligated to the 4.1 kb DNA fragment of BglII-EcoRI-digested pSKTCFPH, plasmid previously produced by ligating PstI-HindIII-digested pBluescript SK+ and 1.4 kb fragment of the PstI-HindIII-digested pcDTCF II. A part of the ligation mixture was used for transformation of *E. coli*, DH5 α . The plasmid DNAs were prepared from ampicillin resistant transformants and the DNA sequences were determined to screen for plasmid pSKTCFPH-2 in which the codon, TCC, for Ser at 399th was replaced with the codon, GCT, for Ala. Plasmid pSKTCFPH-12 was produced by ligating PCR products and the 4.1 kb DNA fragment of the BglII-EcoRI digested plasmid, pSKTCFPH-1 in a similar way. The DNA sequences of the portion of DNAs derived from PCR products in all plasmids were determined to confirm their sequences. Ampicillin was purchased from Sigma Co..

To mutagenize the codon for Ser at 563rd, 25 cycles of PCR were carried out by a mutant primer, TCF-3R 5'-ATACCAGCTGGGCAACATTGAGAAC-3', a wild type primer, TCF1202F 5'-GGCAACTTATCCCAAACAAGATCTGG-3', AmpliTaq polymerase (Takara Co.), and 4 ng of the plasmid pcDTCF II as a template. The PCR products were purified by centricon 100 (Amicon Co.) to remove primers, digested with restriction enzymes, XhoI and PvuII (Takara Co.). pcDTCF II was digested with XhoI and ligated to generate pcDTCF Δ Xho, which lacks the 1.1 kb XhoI DNA fragment. pcDTCF Δ Xho was digested with SphI and PvuII and the 4.8 kb of XhoI-SphI DNA fragment and the 0.5 kb of PvuII-SphI fragment were purified and ligated to XhoI-PvuII-digested PCR products described above by the DNA Ligation Kit. A part of the ligation mixture was used for transformation of *E. coli*, MC1061/P3 (Invitrogen Co.).

The plasmid DNAs were prepared from ampicillin- and tetracycline-resistant transformants and plasmid, pcDTCF Δ XhoI-3 in which the codon, TCC, for Ser at 563rd was replaced with the codon, GCC, for Ala, was selected in a similar way. Tetracycline was purchased from Sigma Co. .

Two rounds of PCR were carried out to mutagenize the codon for Ser at 650th. The first PCRs were carried out independently by AmpliTaq polymerase (Takara Co.), 4 ng of the plasmid pcDTCF II as a template, and a pair of a mutant primer, TCF-4F 5'-ACTCTGAATGAGGCTGAAATATGTG-3' and a wild type primer, TCF2203R 5'-GGCATGCACAGTTGTATTGGTGGGTGCTTCAG-3' or a pair of a mutant primer, TCF-4R 5'-CACATATTTGAGCCTCATTGAGAGT-3' and a wild type primer, TCF1685F 5'-AACAGGTTCTCAATGTTTCCCAG-3'. The PCR products were purified by DE81 paper (Whatman Co.) and one tenth of them was used for the second PCR by the primers, TCF2203R and TCF1685F. The PCR product was separated in an agarose gel, purified by DE81 paper and digested with BglII and SphI. On the other hand, pcDTCF Δ XhoI and pcDTCF Δ XhoI-3 were digested with BglII and SphI and the 4.9 kb of DNA fragments were purified, ligated to the BglII-SphI-digested PCR fragment described above by the DNA Ligation Kit. One tenth of the ligation mixtures was used to transform E. coli, MC1061/P3. The plasmid DNAs were prepared from ampicillin- and tetracycline-resistant transformants and plasmid, pcDTCF Δ XhoI-4 in which the codon, TCT, for Ser at 650th, was replaced with the codon, GCT, for Ala and plasmid, pcDTCF Δ XhoI-34 in which both codons for Ser at 563rd and at 650th were replaced with the codons for Ala, were selected, in a similar way.

③ Construction of expression vectors.

1) Construction of vectors for transient expression.

Plasmids, pSKTCFPH, pSKTCFPH-1, pSKTCFPH-2, and pSKTCFPH-12 were digested with XhoI and 1.1 kb of DNA fragments were purified. Plasmids, pcDTCF Δ XhoI, pcDTCF Δ XhoI-3, pcDTCF Δ XhoI-4, and pcDTCF Δ XhoI-34 were digested with XhoI and ligated to four kinds of 1.1 kb of DNA fragments described above. Fifteen ligation mixtures except the combination of 1.1 kb of pSKTCFPH and pcDTCF Δ XhoI were used to transform E. coli, MC1061/P3. Fifteen expression

vectors, pcDTCF-1, pcDTCF-2, pcDTCF-3, pcDTCF-4, pcDTCF-12, pcDTCF-13, pcDTCF-14, pcDTCF-23, pcDTCF-24, pcDTCF-34, pcDTCF-123, pcDTCF-124, pcDTCF-134, pcDTCF-234, and pcDTCF-1234 were selected. Analysis by restriction enzymes is confirmed the structures of these vectors. When these plasmids are transfected into mammalian cells, for instance COS cells, genes for TCF and the modified TCF are expressed under the control of cytomegalovirus (CMV) promoter. The construction of these plasmids are shown in Figure 5.

2) Construction of vectors for stable expression.

Plasmid pHSG396 (Takara Co.) was digested with HindIII, blunt-ended with the DNA Blunting Kit (Takara Co.), purified with Gene Clean, ligated to NotI linker, 8mer 5'-GCGGCCGC-3' (Takara Co.), and used to transform *E. coli*, DH5 α . The plasmid DNAs were prepared from chloramphenicol resistant transformants and plasmid, pHSG NotI, which did not contain HindIII site but contain NotI site was selected. pHSG NotI, was digested with Sall and SphI and ligated to the 2.3 kb of full-length TCF cDNA digested with Sall and SphI to produce plasmid, pHSG N-TCF. The 1.1 kb of XhoI-XhoI fragment was deleted from the plasmid, pHSG N-TCF, to produce plasmid, pHSG N-TCFII Δ X. pHSG N-TCFII Δ X, was digested with BstPI and SphI and the 2.3 kb of DNA fragment was purified, ligated to the BstPI-SphI-digested 15 plasmids, pcDTCF-1, pcDTCF-2, pcDTCF-3, pcDTCF-4, pcDTCF-12, pcDTCF-13, pcDTCF-14, pcDTCF-23, pcDTCF-24, pcDTCF-34, pcDTCF-123, pcDTCF-124, pcDTCF-134, pcDTCF-234, and pcDTCF-1234, and the BstPI-SphI-digested pcDTCF that contained the wild type TCF cDNA by DNA Ligation Kit, and used to transform *E. coli*, DH5 α . The plasmid DNAs were prepared from chloramphenicol-resistant transformants and the 16 plasmids that contained 2.3 kb of the wild-type TCF or each of the mutant TCF cDNAs in the plasmid, pHSG NotI, were selected.

These plasmids were designated as pHSGN-TCF, pHSGN-TCF-1, pHSGN-TCF-2, pHSGN-TCF-3, pHSGN-TCF-4, pHSGN-TCF-12, pHSGN-TCF-13, pHSGN-TCF-14, pHSGN-TCF-23, pHSGN-TCF-24, pHSGN-TCF-34, pHSGN-TCF-123, pHSGN-TCF-124, pHSGN-TCF-

134, pHSGN-TCF-234, and pHSGN-TCF-1234. These 16 plasmids were digested with Sall and NotI, ligated to the XhoI-NotI-digested expression vector, BCMGSneo, and used to transform E. coli, DH5 α . BCMGSneo is a plasmid that contains the replication origin of bovine papilloma virus and cytomegalovirus promoter, and is capable of replicating in E. coli. BCMGSneo is provided by Dr. H. Karasuyama in Basel Immunology Institute and is described in Idenshikohgaku hand book, p297-299, Yohdo Co., 1991. The plasmid DNAs were prepared from ampicillin resistant transformants and the 16 expression vectors that contained 2.3 kb of wild-type TCF or each of the mutant TCF cDNAs in BCMGSneo were selected. These plasmids were designated as, pBPV-TCF, pBPV-TCF-1, pBPV-TCF-2, pBPV-TCF-3, pBPV-TCF-4, pBPV-TCF-12, pBPV-TCF-13, pBPV-TCF-14, pBPV-TCF-23, pBPV-TCF-24, pBPV-TCF-34, pBPV-TCF-123, pBPV-TCF-124, pBPV-TCF-134, pBPV-TCF-234, and pBPV-TCF-1234. Antibiotics, chloramphenicol was purchased from Sigma Co.. E. coli strains containing pBPV-TCF-3 or pBPV-TCF-13 were deposited to National Institute of Bioscience and Human Technology as FERM BP-4454 and FERM BP-4455.

④ Preparation and purification of the TCF or the modified TCFs expression plasmids.

The 16 E.coli strains containing each of the expression vectors, pBPV-TCF, pBPV-TCF-1, pBPV-TCF-2, pBPV-TCF-3, pBPV-TCF-4, pBPV-TCF-12, pBPV-TCF-13, pBPV-TCF-14, pBPV-TCF-23, pBPV-TCF-24, pBPV-TCF-34, pBPV-TCF-123, pBPV-TCF-124, pBPV-TCF-134, pBPV-TCF-234, and pBPV-TCF-1234, were cultured in 400 ml of medium containing 50 μ g/ml of ampicillin at 37°C. When the absorbance at 600nm of each culture broth reached 0.8, chloramphenicol was added to the broth at a final concentration of 170 μ g/ml and each broth was cultured overnight. These 16 plasmids were prepared by the alkali-SDS method and purified by cesium chloride density gradient ultra centrifugation as described by Maniatis et. al. (Molecular Cloning 2nd ed.).

⑤ Transfection of the TCF- and the modified TCF- expression plasmids into the cultured animal cell lines.

The 16 expression plasmids were transfected into a mouse cell line, C 127 by TRANSFECTAM (IBF Co. Maryland, USA), DNA transfection reagent for cultured mammalian cell lines, as described below. One day prior to transfection, approximately 10^6 cells of mouse C127 were suspended in DME medium (GIBCO Co.) containing 10 % of fetal calf serum and incubated at 37°C overnight in a CO₂ incubator (a humidified incubator in an atmosphere of 5-7% of CO₂), in 25 cm² tissue culture flasks (Sumitomo Bakelite, for adherent cells). (Cells were cultured at 37 °C in a humidified incubator in an atmosphere of 5-7% CO₂, unless otherwise noted.). The cells were washed twice with Opti. MEM medium (GIBCO Co.) before transfection. After adding 2ml of Opti. MEM to the monolayer of the cells, transfection was carried out by 10 µg of the plasmid DNA as described in the manufacturer's protocol. After incubated for 6 hours, 7.5 ml of DME medium was added to the flasks. Then, the cells were incubated for two more days at 37 °C. The medium was replaced with fresh DME medium on the first day. The cells were trypsinized, washed once with DME medium and suspended in 50 mL of DME medium containing 100 µg/ml of G418. One hundred micro-liter of the suspension was added to each well in a 96-well flat-bottomed plates and incubated at 37°C . One week later, 100 µL of DME medium containing 100 µg/ml of G418 was added to each well, and the plates were incubated at 37°C . Another week later, expression of the TCF or the modified TCFs was detected by measuring the concentration of TCF or the modified TCFs in 100 µL of the cultured media by an enzyme immuno assay employing anti-TCF monoclonal antibodies (N. Shima et.al. Gastroenterologia Japonica 26 (4) 477-482, 1991). The cell lines expressing TCF or the modified TCFs were cultured at 37°C in a 12-well tissue culture plate or in a 25 cm² flask according to the cell

numbers. Cell lines which express the modified TCFs (a total of 15 modified TCFs designated as TCF-1, TCF-2, TCF-3, TCF-4, TCF-12, TCF-13, TCF-14, TCF-23, TCF-24, TCF-34, TCF-123, TCF-124, TCF-134, TCF-234, and TCF-1234) were thus obtained.

⑥ Large-scale cultivation of the cell lines which produce TCF or the modified TCFs.

The confluent cells producing TCF or the modified TCFs in 75 cm² tissue culture flasks were harvested by trypsinization, followed by inoculating into three 225 cm² tissue culture flasks. One hundred milliliters of DME medium was added to each flask and the culture was incubated at 37°C. Four days later, the confluent cells were trypsinized and suspended in the DME medium. The cell suspension was diluted 10-fold with DME medium (a total volume of 3 L). One hundred milliliters of the diluted cell suspension was inoculated into a new 225 cm² tissue culture flask. The cells were cultured at 37°C for 5 days in thirty 225 cm² tissue culture flasks. The cultured supernatant (a total volume of 3 L) was collected. Cells were harvested from 6 flasks, followed by inoculating into sixty 225cm² tissue culture flasks. To each flask, 100 ml of the medium was added and incubated at 37 °C for 5 days. The cultured supernatant (a total volume of 6 L) was collected. Thus, 9 L of the cultured supernatant which contains the TCF or the modified TCFs was obtained.

⑦ Purification of the TCF and the modified TCFs.

A three-step purification was performed as described below.

1) Heparin-sepharose CL-6B

Nine liters of the cultured supernatant which contains each of the modified TCF centrifuged at 6,000 rpm for 30 min. to remove insoluble materials. The supernatant was applied at an approximate flow rate of 200ml per

hour to a heparin-sepharose CL-6B column (2.5 X 12 cm) (Pharmacia Co.) which had been equilibrated with 300 mL of the equilibration buffer, 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M of NaCl and 0.01% of Tween 20. The column was then washed with approximately 700 mL of the equilibration buffer. The TCF or the modified TCFs were eluted with 10mM Tris-HCl buffer (pH 7.5) containing 2 M NaCl and 0.01% of Tween 20 and the fractions of 3 mL were collected. The fractions were monitored by the absorbance at 280 nm and the fractions containing TCF or the modified TCFs (approximately 100 mL) were obtained.

2) Mono S-FPLC

The eluate which contains TCF or the modified TCFs was dialyzed against 10 mM of phosphate buffer (pH 6.5) containing 0.15 M of NaCl and centrifuged at 12,000 rpm for 90 min. to remove insoluble materials. The supernatant was applied at a flow rate of 1 mL/min. to a Mono S column (0.5 X 5 cm, Pharmacia, FPLC) which had been equilibrated with approximately 20 ml of 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.01 % Tween 20 (buffer A). The column was washed once with approximately 30 mL of buffer A. Then the TCF or the modified TCFs were eluted at a flow rate of 0.5 mL/min. from the column with a linear gradient of NaCl (up to 1.0 M) and fractions of 0.5 ml were collected. The fractions containing TCF or the modified TCFs (an approximate volume of 4mL) which were eluted with 0.7-0.8 M NaCl were obtained.

3) Heparin 5-PW-FPLC

Two volumes (8 mL) of 10 mM Tris-HCl (pH 7.5) containing 0.01 % Tween 20 were added to the eluate which contains TCF or the modified TCFs. This diluted solution was applied at a flow rate of 1 mL/min. to a heparin 5-PW column (0.5 X 7.5 cm, TOSO Co., FPLC) which had been equilibrated with approximately 20 mL of 10 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl and 0.01 % Tween 20 (buffer B). The column was washed with approximately 30 ml of buffer B. Then TCF or the modified TCFs were eluted at a flow rate of 0.5 mL/min. with a linear

gradient of NaCl (up to 2.0 M) and fractions of 0.5 mL were collected. The fractions containing TCF or the modified TCFs (those eluted with approximately 1.3 M NaCl, a total volume of 3 mL) were obtained. These fractions were dialyzed against deionized water, lyophilized and reconstituted with phosphate-buffered saline (PBS) containing 0.001% Tween 20. The yield and the recovery of each of the final purified modified TCFs are shown in Table 1. The yields were determined with polyclonal EIA described in the following section.

Table 1 : The yield and the recovery of each of the final purified modified TCFs

Name	Yield(μ g)	Recovery(%)
TCF	2760	31
TCF-1	872	17
TCF-2	920	40
TCF-3	253	16
TCF-4	560	16
TCF-12	688	33
TCF-13	1088	23
TCF-14	350	27
TCF-23	810	28
TCF-24	648	19
TCF-34	668	34
TCF-123	340	11
TCF-124	400	23
TCF-134	187	22
TCF-234	400	15
TCF-1234	155	8

⑧ Quantitation of the purified modified TCFs.

1) Preparation of polyclonal antibodies and labeling of the antibodies.

Anti-TCF antiserum was obtained from rabbits which were immunized with TCF. The anti-TCF IgG was purified from the antiserum by an Affi-Gel protein A Sepharose (Bio Rad Co.) according to the manufacturer's protocol. The purified IgG was dialyzed overnight against PBS, and applied at a flow rate of 0.5 mL/min. to a TCF affinity column in which TCF was immobilized to affigel 10 (Bio Rad Co.). The immobilized column was washed with PBS and the anti-TCF IgG was eluted with 0.1 M Glycine-HCl buffer (pH 2.5). The eluate was dialyzed against PBS and the purified anti-TCF polyclonal antibodies were thus obtained. The peroxylase labeled antibodies were prepared as described by Ishikawa et al. (J. Immunoassay, vol. 4, 209-327, 1983).

2) Quantitation of the purified modified TCFs.

Anti-TCF antibodies were dissolved at a concentration of 10 μ g/mL in 0.1 M NaHCO₃. The antibody solution was added (100 μ L/well) to 96-well microtiter plates (NUNC Co.) and left overnight at room temperature. This step allowed the antibodies to attach to the plates. Block Ace (Snow Brand Milk Products Co.Ltd.) diluted two-fold with deionized water was added to the antibody-coated microtiter-well plates (200 μ L/ml) and left for an hour at room temperature for blocking each well. The plates were then washed three times with PBS containing 0.1% Tween 20 (washing buffer). The modified TCF were diluted with appropriate volume of the first buffer (0.2 M Tris-HCl pH 7.4 containing 40% Block Ace and 0.1% Tween 20) to prepare modified TCF samples. Standard TCF solution was prepared by sequentially diluting 10 ng/mL of TCF solution with the first buffer. One hundred micro liters of the modified TCF samples were added to each well, left for 3 hours at 37 °C and washed three times with the washing buffer. The Peroxylase-conjugated antibody solution was diluted 400-fold with the second buffer (0.1 M Tris-HCl buffer pH 7.4

containing 20% Block Ace, 0.1% Tween 20 and 0.5 mg/mL of mouse IgG). One hundred microliters of the diluted peroxidase-labeled antibody solution was added to each well, left for 2 hours at 37 °C and washed three times with the washing buffer. Subsequently, 100 μ L of substrate solution (0.4 mg/mL of o-phenylene diamine dihydrochloride and 0.006% H₂O₂ in 0.1 M citrate-phosphate buffer, pH 4.5) was added to each well and incubated at 37°C for 30 minutes in a dark place. The enzyme reaction was stopped by addition of 50 μ L of 6N H₂SO₄. The absorbance at 492 nm was measured on an immuno reader (Corona Co.).

⑨ Analysis of the purified TCF or the modified TCFs on SDS-polyacrylamide gel electrophoresis.

Five micrograms of each of purified modified TCF was subjected to an SDS-polyacrylamide gel. Modified TCFs (TCF-3 and TCF-13) with prolonged biological half-lives as described later, and the wild-type TCF were applied to an SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 7. Electrophoresis was performed either in the presence (reducing conditions) or absence (non-reducing conditions) of β -mercaptoethanol. As indicated in Fig. 7 TCF showed two adjacent bands with approximate molecular masses of 78,000 and 74,000 under non-reducing conditions. The two bands of TCF-3 migrated faster than those of TCF, and the two bands of TCF-13 migrated faster than those of TCF-3, under non-reducing conditions. Under the reducing conditions, three protein bands with approximate molecular masses of 52,000, 30,000 and 26,000 were observed for TCF. Similarly, three bands with 52,000, 26,000 and 22,000 for TCF-3, and three bands with 48,000, 26,000 and 22,000 for TCF-13 were observed. The decrease in molecular mass of these modified TCFs was probably due to the removal of oligosaccharide chains. The results suggested that the desired modified TCFs were obtained. No other protein bands were detected except those deduced from the structure of the two modified proteins.

Example 2

Biological activities of the TCF and the modified TCFs *in vitro*.

① Growth stimulating activity for hepatocytes.

Growth stimulating activity for hepatocytes were determined as follows. Rat hepatocytes were isolated from a Wister rat (body weight was approximately 200 g) by the method of Seglen (Methods in cell biology Vol.13, p.29, Academic Press, New York). The basal medium, Williams E (Flow laboratories Co.), containing 10% fetal bovine serum and $10\mu\text{M}$ dexamethasone) was used for the growth of the cells. One hundred microliters of the basal medium containing 1.0×10^4 cells was added to each well in a 96-well flat-bottomed plate (Falcon Co.) and incubated at 37°C . After 24 hours of incubation, $100\mu\text{L}$ of the basal medium containing TCF or the modified TCFs was added to each well and incubated for 22 hours at 37°C . Each well was supplemented with $1\mu\text{Ci}$ of ^3H -thymidine (Amersham Co.) and further incubated at 37°C for 2 hours. After washing twice with cold PBS, cells were trypsinized with 0.5% trypsin and harvested on a sheet of glass filter by a cell harvester. Radioactivities incorporated into the cells in each well were counted by Matrix 96 (Packard Co.). As shown in Fig. 8, all the modified TCFs were shown to maintain growth stimulating activities for rat hepatocytes.

② Tumor cytotoxic activity.

Tumor cytotoxic activities of TCF, TCF-3 and TCF-13 were measured. Meth A sarcoma was used as a target cell line. The cells were suspended in RPMI medium (GIBCO Co.) supplemented with 10% FCS at a final cell density of 2×10^4 cells per mL. Fifty microliters of the cell suspension was inoculated into each

well in 96-well flat-bottomed microtiter plates. The purified TCF, TCF-3 and TCF-13 were serially diluted with RPMI medium from the concentration of 50 ng/mL, and the 50 μ L of each diluted sample was added to each test well. After incubation at 37°C for 5 days, MTT was added at a final concentration of 0.5 mg/ml and the plates were incubated at 37°C. Four hours later, 100 μ L of the solution containing 10% SDS and 0.01 M NH_4Cl was added to each well and left overnight at room temperature. Next day, the absorbance at 620 nm was measured as a parameter of the viable cell numbers in each well. Fig. 9 shows the results of the assay. TCF, TCF-3 and TCF-13 having cytotoxic activities to Meth A cells in a dose-dependent manner.

Example 3

Measurement of biological half-lives of the TCF and the modified TCFs *in vivo*.

Anti-TCF polyclonal antibody was dissolved in 0.1 M NaHCO_3 solution to a final concentration of 10 μ g/mL. One hundred microliters of the antibody solution was added to each well and the plates were left overnight at room temperature. The wells were filled with 50% solution of Block Ace in H_2O , incubated for 1 hour at room temperature and washed three times with washing solution (PBS containing 0.1% Tween 20). Plasma samples periodically collected from the rats which were intravenously injected with TCF or the modified TCFs were diluted with normal rat serum, when necessary. TCF solutions serially diluted with normal rat serum (from 10 ng/mL) was used as the standard TCF solution. Fifty microliters of the sample solution and 50 μ L of the first buffer (0.2 M Tris HCl pH 7.3 containing 50% Block Ace, 0.2 M NaCl, 0.1% Tween 20, 0.2% CHAPS, 20 mM Benzamidine hydrochloride, and 10 mM EDTA) was mixed and added to each well. The plates were left for 3 hours at 37 °C and washed three

times with the washing buffer. One hundred microliters of the peroxidase-labeled anti-TCF antibody solution, which was 400-fold diluted with 0.1 M phosphate buffer pH7.0 containing 10 % Block Ace, 0.15 M NaCl, 0.1% Tween 20, 4% rat serum and 0.5% mouse IgG, was added to each well. Plates were incubated for 2 hours at 37 °C and washed three times with the washing buffer. Subsequently, 100 μ L of substrate solution (0.4 mg/mL of o-phenylene diamine dihydrochloride and 0.006% H₂O₂ in 0.1 M citrate-phosphate buffer, pH 4.5) was added to each well and incubated at 37°C for 30 minutes in a dark place. The enzyme reaction was stopped by addition of 50 μ L of 6N H₂SO₄. The absorbance at 492 nm was measured on an immuno reader (Corona Co.). Thus concentrations of the TCF or the modified TCFs in rat serum were determined.

Time course of plasma levels were examined by EIA in rats after the single intravenous injection of TCF and the modified TCFs. Male Wister rats weighing about 200 g were used. After the intravenous injection of TCF to the rats, the plasma level declined biexponentially, well described by a two-compartment model. Plasma half-lives of rapid phase and slower phase were 2.4 ± 2.5 min. ($t'_{1/2\alpha}$) and 15.6 ± 4.6 min. ($t'_{1/2\beta}$) in rats after the TCF injection at a dose of 50 μ g/kg, respectively. Plasma level profiles of modified TCF were similar to that of the wild-type TCF, but their plasma levels declined slower than that of the wild-type TCF after the intravenous injection at the same dose.

As shown in Table 2, plasma half-lives of TCF-3 and TCF-13 were prolonged and their total clearance were decreased. TCF-3 and TCF-13 had larger AUC(the area under the plasma concentration - time curve) compared to the wild-type TCF.

Table 2 : Pharmacokinetics parameters of the modified TCFs

SAMPLE	$t_{1/2} \alpha$ (min)	$t_{1/2} \beta$ (min)
TCF	2.4 ± 0.5	15.6 ± 4.6
TCF-1	2.4 ± 0.3	19.6 ± 0.3
TCF-2	2.2 ± 0.6	18.2 ± 2.5
TCF-3	2.9 ± 0.3	$54.2 \pm 8.3^{**}$
TCF-4	2.7 ± 0.1	20.6 ± 3.3
TCF-12	2.7 ± 0.3	18.1 ± 1.6
TCF-13	$3.8 \pm 0.5^{**}$	$24.9 \pm 3.1^*$
TCF-14	1.9 ± 0.4	18.8 ± 2.9
TCF-23	2.4 ± 0.5	15.4 ± 0.5
TCF-24	2.3 ± 0.1	16.9 ± 1.7
TCF-34	2.2 ± 0.5	18.0 ± 2.1
TCF-123	2.2 ± 0.1	17.2 ± 0.8
TCF-124	2.3 ± 0.1	15.7 ± 2.7
TCF-134	2.3 ± 0.4	19.1 ± 3.2
TCF-234	2.6 ± 0.6	16.8 ± 1.6
TCF-1234	$2.9 \pm 0.1^*$	$24.2 \pm 5.5^*$

Mean \pm SD

Significantly difference from Wild-type TCF (*P<0.05, ** P<0.01)

Table 3 : Pharmacokinetics parameters of the modified TCFs

SAMPLE	AUC (ng · min/ml)	CL _{total} (ml/min/kg)
TCF	1030.0 ± 257.9	51.5 ± 13.9
TCF-1	811.6 ± 158.3	63.2 ± 12.2
TCF-2	1143.7 ± 551.7	49.8 ± 18.9
TCF-3	679.5 ± 292.1**	18.8 ± 2.1 **
TCF-4	897.8 ± 263.5	58.6 ± 14.7
TCF-12	1246.4 ± 290.8	41.7 ± 10.6
TCF-13	301.0 ± 279.7**	6.0 ± 0.2 **
TCF-14	972.5 ± 162.2	52.5 ± 9.5
TCF-23	1223.0 ± 356.7	43.6 ± 14.3
TCF-24	1017.8 ± 210.0	51.0 ± 8.3
TCF-34	983.0 ± 61.0	51.0 ± 3.2
TCF-123	963.8 ± 66.2	52.0 ± 3.5
TCF-124	1126.3 ± 265.0	45.9 ± 9.5
TCF-134	960.9 ± 279.7	55.2 ± 16.7
TCF-234	892.9 ± 119.7	56.6 ± 7.1
TCF-1234	1266.5 ± 78.9	39.6 ± 2.6

Mean ± SD

Significantly difference from Wild-type TCF (*P<0.05, ** P<0.01)

These results indicated that TCF-3 and TCF-13 had slower metabolic fates and have larger bioavailabilities than those of the wild-type TCF. The amino acid

sequence of TCF-3 discloses in Seq. Id No.2, and the sequence of TCF-13 discloses in Seq. Id No. 3.

The modified TCFs in the present invention have longer biological half-lives, maintaining growth stimulating activities for hepatocytes and cytotoxic activities to tumor cells, and are therefore useful as therapeutic agents for liver diseases or as anti-cancer drugs.

REFERENCE OF MICROORGANISM

(1) pcTCF(S)/MC1061/P3

Organization of Deposition:

National Institute of Bioscience and Human-Technology,
Agency of Industrial Science and Technology,
Ministry of International Trade and Industry

Address:

1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan

Deposition Number:

FERM BP-3479

(2) pBPV-TCF-3

Organization of Deposition:

National Institute of Bioscience and Human-Technology,
Agency of Industrial Science and Technology,
Ministry of International Trade and Industry

Address:

1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan

Deposition Number:

FERM BP-4454

(3) pBPV-TCF-13

Organization of Deposition:

National Institute of Bioscience and Human-Technology,
Agency of Industrial Science and Technology,
Ministry of International Trade and Industry

Address:

1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan

Deposition Number:

FERM BP-4455

SEQUENCE LISTING

Seq Id No. 1

Length: 723

Type: amino acid

Molecule Type: protein

Sequence

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Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys Gln Cys
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 595 600 605

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 Pro Gln Ser

Seq Id No.2

Length: 723

Type: amino acid

Molecule Type: protein

Sequence

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Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys
           130          135          140
Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His Glu His
145          150          155          160
Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr Cys Arg Asn Pro Arg
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 225 230 235 240
 Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp Asp Asn Tyr Cys Arg
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 Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr Met Gly Asn Leu Ser Gln
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 Gln Cys Phe Pro Ser Arg Asp Leu Lys Asp Tyr Glu Ala Trp Leu Gly
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Asn Val Ala Gln Leu Val Tyr Gly Pro Glu Gly Ser Asp Leu Val Leu
 565 570 575
 Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp Phe Val Ser Thr Ile
 580 585 590
 Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro Glu Lys Thr Ser Cys Ser
 595 600 605
 Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn Tyr Asp Gly Leu Leu
 610 615 620

Arg Val Ala His Leu Tyr Ile Met Gly Asn Glu Lys Cys Ser Gln His
625 630 635 640
His Arg Gly Lys Val Thr Leu Asn Glu Ser Glu Ile Cys Ala Gly Ala
 645 650 655
Glu Lys Ile Gly Ser Gly Pro Cys Glu Gly Asp Tyr Gly Gly Pro Leu
 660 665 670
Val Cys Glu Gln His Lys Met Arg Met Val Leu Gly Val Ile Val Pro
 675 680 685
Gly Arg Gly Cys Ala Ile Pro Asn Arg Pro Gly Ile Phe Val Arg Val
 690 695 700
Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile Ile Leu Thr Tyr Lys Val
705 710 715 720
Pro Gln Ser

Seq Id No.3

Length: 723

Type: amino acid

Molecule Type: protein

Sequence

Met	Trp	Val	Thr	Lys	Leu	Leu	Pro	Ala	Leu	Leu	Leu	Gln	His	Val	Leu
1				5					10					15	
Leu	His	Leu	Leu	Leu	Leu	Pro	Ile	Ala	Ile	Pro	Tyr	Ala	Glu	Gly	Gln
				20				25					30		
Arg	Lys	Arg	Arg	Asn	Thr	Ile	His	Glu	Phe	Lys	Lys	Ser	Ala	Lys	Thr
				35				40					45		
Thr	Leu	Ile	Lys	Ile	Asp	Pro	Ala	Leu	Lys	Ile	Lys	Thr	Lys	Lys	Val
				50				55					60		
Asn	Thr	Ala	Asp	Gln	Cys	Ala	Asn	Arg	Cys	Thr	Arg	Asn	Lys	Gly	Leu
65					70					75				80	
Pro	Phe	Thr	Cys	Lys	Ala	Phe	Val	Phe	Asp	Lys	Ala	Arg	Lys	Gln	Cys
				85					90					95	
Leu	Trp	Phe	Pro	Phe	Asn	Ser	Met	Ser	Ser	Gly	Val	Lys	Lys	Glu	Phe
				100					105					110	
Gly	His	Glu	Phe	Asp	Leu	Tyr	Glu	Asn	Lys	Asp	Tyr	Ile	Arg	Asn	Cys
				115				120						125	
Ile	Ile	Gly	Lys	Gly	Arg	Ser	Tyr	Lys	Gly	Thr	Val	Ser	Ile	Thr	Lys
				130				135						140	
Ser	Gly	Ile	Lys	Cys	Gln	Pro	Trp	Ser	Ser	Met	Ile	Pro	His	Glu	His
145					150					155				160	
Ser	Tyr	Arg	Gly	Lys	Asp	Leu	Gln	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	Arg
				165					170					175	

Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr Ser Asn Pro Glu Val Arg
 180 185 190
 Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser Glu Val Glu Cys Met Thr
 195 200 205
 Cys Asn Gly Glu Ser Tyr Arg Gly Leu Met Asp His Thr Glu Ser Gly
 210 215 220
 Lys Ile Cys Gln Arg Trp Asp His Gln Thr Pro His Arg His Lys Phe
 225 230 235 240
 Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp Asp Asn Tyr Cys Arg
 245 250 255
 Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys Tyr Thr Leu Asp Pro His
 260 265 270
 Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr Cys Ala Asp Asn Thr Met
 275 280 285
Gln Asp Thr Asp Val Pro Leu Glu Thr Thr Glu Cys Ile Gln Gly Gln
 290 295 300
 Gly Glu Gly Tyr Arg Gly Thr Val Asn Thr Ile Trp Asn Gly Ile Pro
 305 310 315 320
 Cys Gln Arg Trp Asp Ser Gln Tyr Pro His Glu His Asp Met Thr Pro
 325 330 335
 Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn Tyr Cys Arg Asn Pro
 340 345 350
 Asp Gly Ser Glu Ser Pro Trp Cys Phe Thr Thr Asp Pro Asn Ile Arg
 355 360 365
 Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys Asp Met Ser His Gly Gln
 370 375 380
 Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr Met Gly Asn Leu Ser Gln
 385 390 395 400

Thr Arg Ser Gly Leu Thr Cys Ser Met Trp Asp Lys Asn Met Glu Asp
 405 410 415
 Leu His Arg His Ile Phe Trp Glu Pro Asp Ala Ser Lys Leu Asn Glu
 420 425 430
 Asn Tyr Cys Arg Asn Pro Asp Asp Asp Ala His Gly Pro Trp Cys Tyr
 435 440 445
 Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr Cys Pro Ile Ser Arg Cys
 450 455 460
 Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu Asp His Pro Val Ile
 465 470 475 480
 Ser Cys Ala Lys Thr Lys Gln Leu Arg Val Val Asn Gly Ile Pro Thr
 485 490 495
 Arg Thr Asn Ile Gly Trp Met Val Ser Leu Arg Tyr Arg Asn Lys His
 500 505 510
 Ile Cys Gly Gly Ser Leu Ile Lys Glu Ser Trp Val Leu Thr Ala Arg
 515 520 525
 Gln Cys Phe Pro Ser Arg Asp Leu Lys Asp Tyr Glu Ala Trp Leu Gly
 530 535 540
 Ile His Asp Val His Gly Arg Gly Asp Glu Lys Cys Lys Gln Val Leu
 545 550 555 560
Asn Val Ala Gln Leu Val Tyr Gly Pro Glu Gly Ser Asp Leu Val Leu
 565 570 575
 Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp Phe Val Ser Thr Ile
 580 585 590
 Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro Glu Lys Thr Ser Cys Ser
 595 600 605
 Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn Tyr Asp Gly Leu Leu
 610 615 620

Arg Val Ala His Leu Tyr Ile Met Gly Asn Glu Lys Cys Ser Gln His
625 630 635 640
His Arg Gly Lys Val Thr Leu Asn Glu Ser Glu Ile Cys Ala Gly Ala
 645 650 655
Glu Lys Ile Gly Ser Gly Pro Cys Glu Gly Asp Tyr Gly Gly Pro Leu
 660 665 670
Val Cys Glu Gln His Lys Met Arg Met Val Leu Gly Val Ile Val Pro
 675 680 685
Gly Arg Gly Cys Ala Ile Pro Asn Arg Pro Gly Ile Phe Val Arg Val
 690 695 700
Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile Ile Leu Thr Tyr Lys Val
705 710 715 720
Pro Gln Ser

CLAIMS

1. The modified TCFs which are characterized by that the amino-acid residues responsible for binding of N-linked oligosaccharide chains are modified so that at least one of the N-linked oligosaccharide chains is prevented from binding to TCF.
2. The modified TCFs according to claim 1, in which one of the residues responsible for N-glycosylation is substituted with other amino-acid residues so that the N-linked oligosaccharide chain(s) are prevented from binding to Asn residue(s) at positions 289th, 397th, 561stst and/or 648th from N-terminal in its amino-acid sequence described in Sequence 1.
3. The modified TCF according to claim 1, in which Ser at position 563rd is substituted with Ala.
4. The modified TCF according to claim 1, in which Asn at position 289th is substituted with Gln, and Ser at position 563rd is substituted with Ala.
5. Modified TCF, a glycoprotein to which DNA sequence encoding the amino-acid sequence described in Sequence 2 is expressed.
6. Modified TCF, a glycoprotein to which DNA sequence encoding the amino-acid sequence described in Sequence 3 is expressed.

Fig. 1

Met Trp Val Thr Lys Leu Leu Pro Ala Leu Leu Leu Gln His Val Leu Leu His Leu Leu Leu Leu Pro Ile Ala Ile Pro Tyr Ala Glu
 Gly Gln Arg Lys Arg Arg Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys Thr Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys
 Thr Lys Lys Val Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys Gly Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp
 Lys Ala Arg Lys Gln Cys Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys Glu Phe Gly His Glu Phe Asp Leu Tyr Glu
 Asn Lys Asp Tyr Ile Arg Asn Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys Ser Gly Ile Lys Cys Gln
 Pro Trp Ser Ser Met Ile Pro His Glu His Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr Cys Arg Asn Pro Arg Gly Glu Glu Gly
 Gly Pro Trp Cys Phe Thr Ser Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser Glu Val Glu Cys Met Thr Cys Asn
 Gly Glu Ser Tyr Arg Gly Leu Met Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln Thr Pro His Arg His Lys Phe
 Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys Tyr Thr Leu Asp
 Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr Thr Glu Cys
 Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn Thr Ile Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro His
 Glu His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys
 Phe Thr Thr Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn
 Gly Lys Asn Tyr Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met Trp Asp Lys Asn Met Glu Asp Leu His Arg His
 Ile Phe Trp Glu Pro Asp Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Ala His Gly Pro Trp Cys Tyr Thr Gly
 Asn Pro Leu Ile Pro Trp Asp Tyr Cys Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu Asp His Pro Val Ile
 Ser Cys Ala Lys Thr Lys Gln Leu Arg Val Val Asn Gly Ile Pro Thr Arg Thr Asn Ile Gly Trp Met Val Ser Leu Arg Tyr Arg Asn
 Lys His Ile Cys Gly Gly Ser Leu Ile Lys Glu Ser Trp Val Leu Thr Ala Arg Gln Cys Phe Pro Ser Arg Asp Leu Lys Asp Tyr Glu
 Ala Trp Leu Gly Ile His Asp Val His Gly Arg Gly Asp Glu Lys Cys Lys Gln Val Leu Asn Val Ser Gln Leu Val Tyr Gly Pro Glu
 Gly Ser Asp Leu Val Leu Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp Phe Val Ser Thr Ile Asp Leu Pro Asn Tyr Gly Cys Thr
 Ile Pro Glu Lys Thr Ser Cys Ser Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn Tyr Asp Gly Leu Leu Arg Val Ala His Leu Tyr
 Ile Met Gly Asn Glu Lys Cys Ser Gln His His Arg Gly Lys Val Thr Leu Asn Glu Ser Glu Ile Cys Ala Gly Ala Glu Lys Ile Gly
 Ser Gly Pro Cys Glu Gly Asp Tyr Gly Gly Pro Leu Val Cys Glu Gln His Lys Met Arg Met Val Leu Gly Val Ile Val Pro Gly Arg
 Gly Cys Ala Ile Pro Asn Arg Pro Gly Ile Phe Val Arg Val Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile Ile Leu Thr Tyr Lys Val
 Pro Gln Ser

Fig. 2

----- TAGGCAC TGACTCCGAA
 CAGGATTCTT TCACCCAGGC ATCTCCTCCA GAGGGATCCG CCAGCCCGTC CAGCAGCACC
ATCTGGGTGA CCAAATCCTT GCCAGCCCTG CTGCTGCAGC ATGTCCTCCT GCATCTCCTC
 CTGCTCCCCA TCGCCATCCC CTATGCAGAG GGACAAAGGA AAAGAAGAAA TACAATTCAT
 GAATTCAAAA AATCAGCAAA GACTACCCCTA ATCAAAATAG ATCCAGCACT GAAGATAAAA
 ACCAAAAAAG TGAATACTGC AGACCAATGT GCTAATAGAT GTACTAGGAA TAAAGGACTT
 CCATTCACCTT GCAAGGCTTT TGTTTTGTAT AAAGCAAGAA AACAATGCCT CTGGTTCCCC
 TTCAATAGCA TGTCAAGTGG AGTGAAAAAA GAATTTGGCC ATGAATTTGA CCTCTATGAA
 AACAAAGACT ACATTAGAAA CTGCATCATT GGTAAAGGAC GCAGCTACAA GCGAACAGTA
 TCTATCACTA AGAGTGGCAT CAAATGTCAG CCCTGGAGTT CCATGATACC ACACGAACAC
 AGCTATCGGG GTAAAGACCT ACAGGAAAAAC TACTGTGGA ATCCTCGAGG GGAAGAAGGG
 GGACCCTGGT GTTTCACAAG CAATCCAGAG GTACGCTACG AAGTCTGTGA CATTCTCAG
 TGTTCAGAAC TTGAATGCAT GACCTGCAAT GGGGAGAGTT ATCGAGGTCT CATGGATCAT
 ACAGAATCAG GCAAGATTTG TCAGCGCTGG GATCATCAGA CACCACACCG GCACAAATTC
 TTGCTGAAA GATATCCCGA CAAGGGCTTT GATGATAATT ATTGCCGCAA TCCCGATGGC
 CAGCCGAGGC CATGGTGCTA TACTCTTGAC CCTCACACCC GCTGGGAGTA CTGTGCAATT
 AAAACATGCG CTGACAATAC TATGAATGAC ACTGATGTTT CTTTGGAAAC AACTGAATGC
 ATCCAAGGTC AAGGAGAAGG CTACAGGGGC ACTGTCAATA CCATTTGGAA TGGAATTCCA
 TGTGAGCGTT GGGATTCTCA GTATCCTCAC GAGCATGACA TGACTCCTGA AAATTTCAAG
 TGCAAGGACC TACGAGAAAA TTACTGCCGA AATCCAGATG GGTCTGAATC ACCCTGGTGT
 TTTACCACTG ATCCAAACAT CCGAGTTGGC TACTGCTCCC AAATTCCTAAA CTGTGATATG
 TCACATGGAC AAGATTGTTA TCGTGGGAAT GGCAAAAATT ATATGGGCAA CTTATCCCAA
 ACAAGATCTG GACTAACATG TTCAATGTGG GACAAGAACA TGGAAGACTT ACATCGTCAT
 ATCTTCTGGG AACCAGATGC AAGTAAGCTG AATGAGAATT ACTGCCGAAA TCCAGATGAT
 GATGCTCATG GACCCTGGTG CTACACGGGA AATCCACTCA TTCCTTGGGA TTATTGCCCT
 ATTTCTCGTT GTGAAGGTGA TACCACACCT ACAATAGTCA ATTTAGACCA TCCCGTAATA
 TCTTGTGCCA AAACGAAACA ATTGGGAGTT GTAAATGGGA TTCCAACACG AACAAACATA
 GGATGGATGG TTAGTTTGGG ATACAGAAAT AAACATATCT GCGGAGGATC ATTGATAAAG
 GAGAGTTGGG TTCTTACTGC ACGACAGTGT TTCCCTTCTC GAGACTTGAA AGATTATGAA
 GCTTGGCTTG GAATTCATGA TGTCCACGGA AGAGGAGATG AGAAATGCAA ACAGGTTCTC
 AATGTTTCCC AGCTGGTATA TGGCCCTGAA GGATCAGATC TGGTTTTAAT GAAGCTTGCC
 AGGCCTGCTG TCCTGGATGA TTTTGTAGT ACGATTGATT TACCTAATTA TGGATGCACA
 ATTCCTGAAA AGACCAGTTG CAGTGTTTAT GGCTGGGGCT AACTGGATT GATCAACTAT
 GATGGCCTAT TACGAGTGGC ACATCTCTAT ATAATGGGAA ATGAGAAATG CAGCCAGCAT
 CATCGAGGGA AGGTGACTCT GAATGAGTCT GAAATATGTG CTGGGGCTGA AAAGATTGGA
 TCAGGACCAT GTGAGGGGGA TTATGGTGGC CCACCTGTTT GTGAGCAACA TAAAATGAGA
 ATGGTTCTTG GTGTCAATTG TCCTGGTCTG GGATGTGCCA TTCCAAATCG TCCTGGTATT
 TTTGTCCGAG TAGCATATTA TGCAAAATGG ATACACAAAA TTATTTTAAAC ATATAAGGTA
 CCACAGTCAT AGCTGAAGTA AGTGTGTCTG AAGCACCAC CAATACAAC GT

Fig. 3

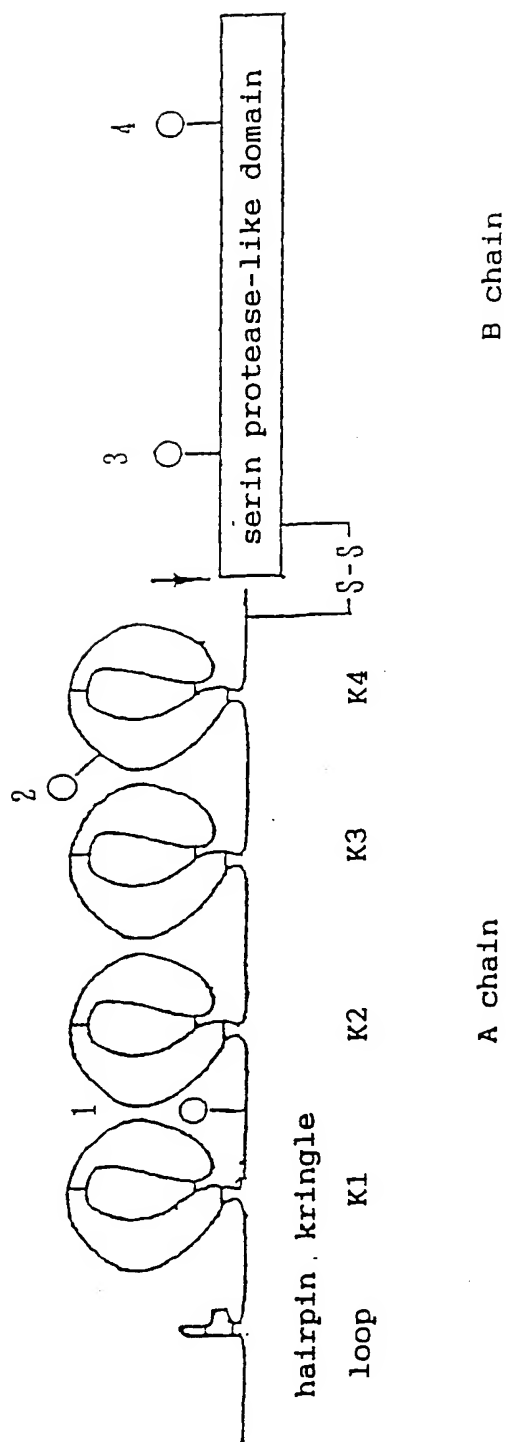


Fig. 4

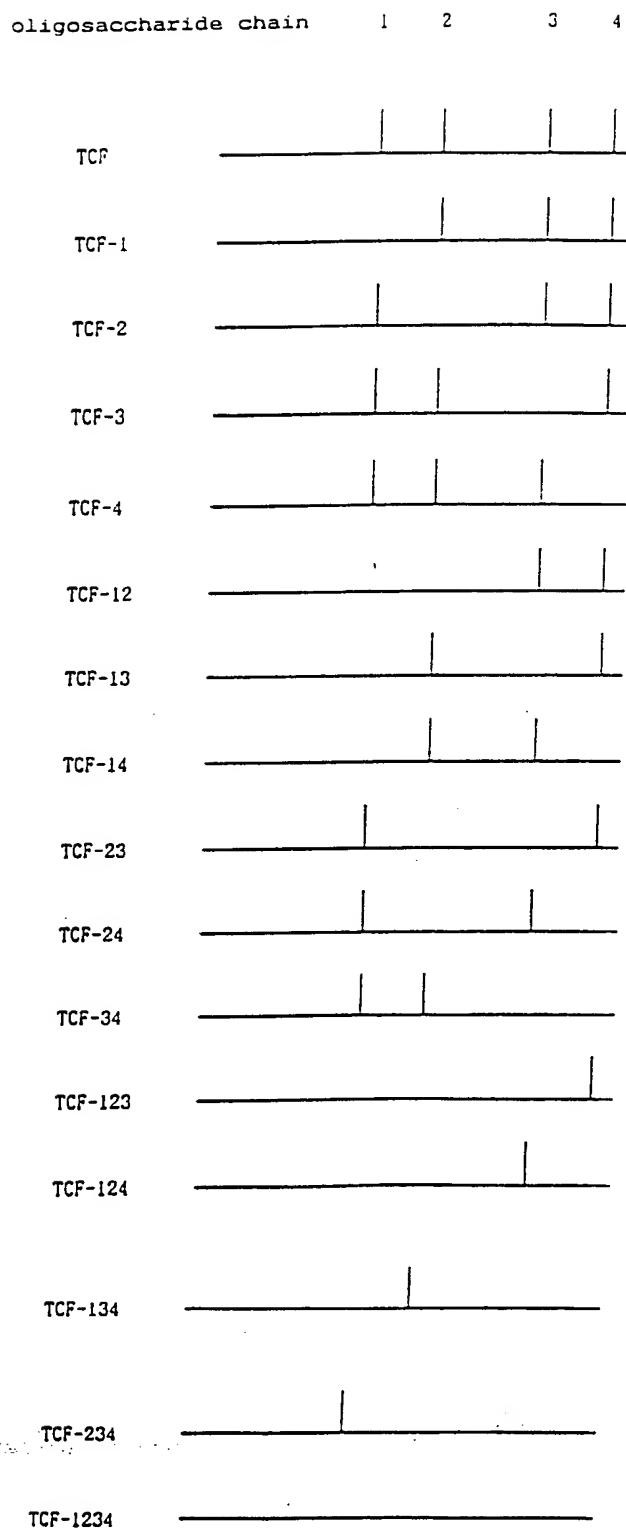


Fig. 5

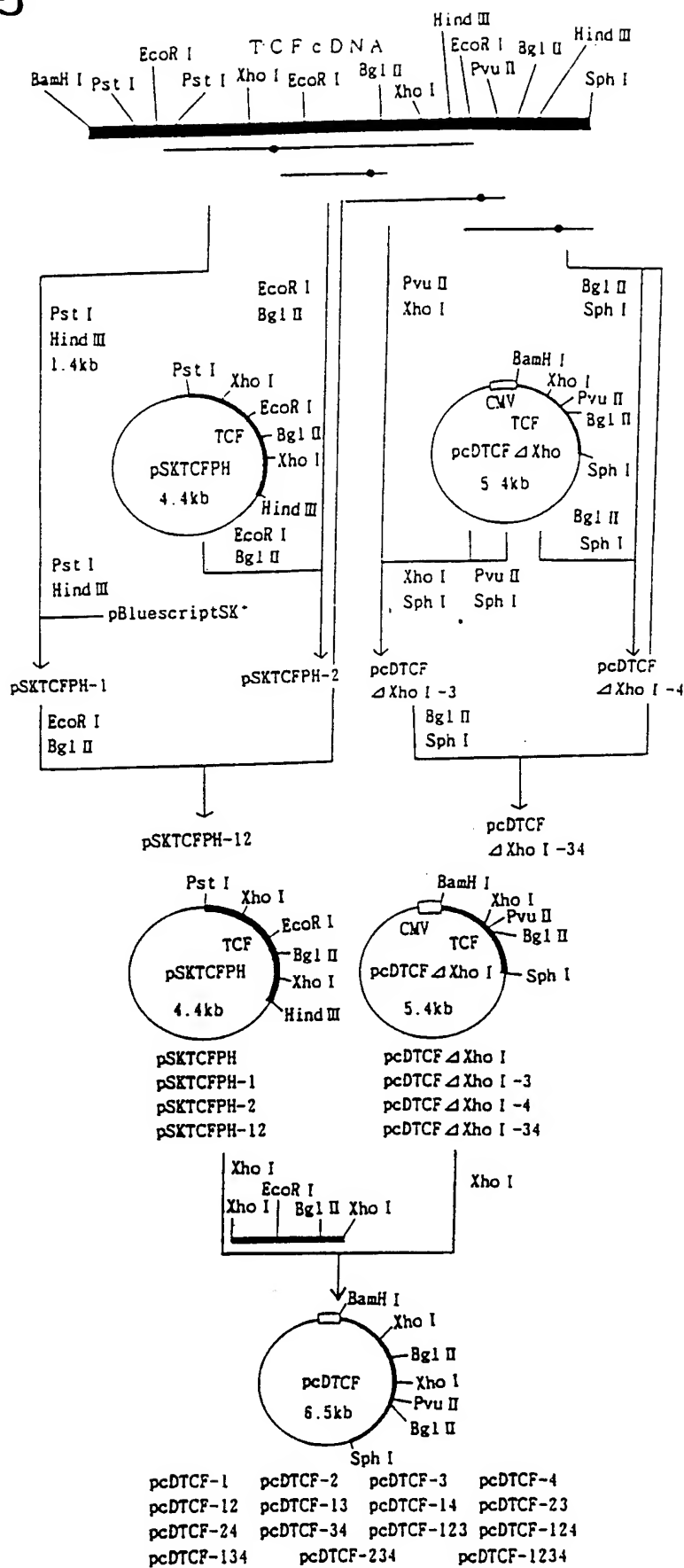


Fig. 6

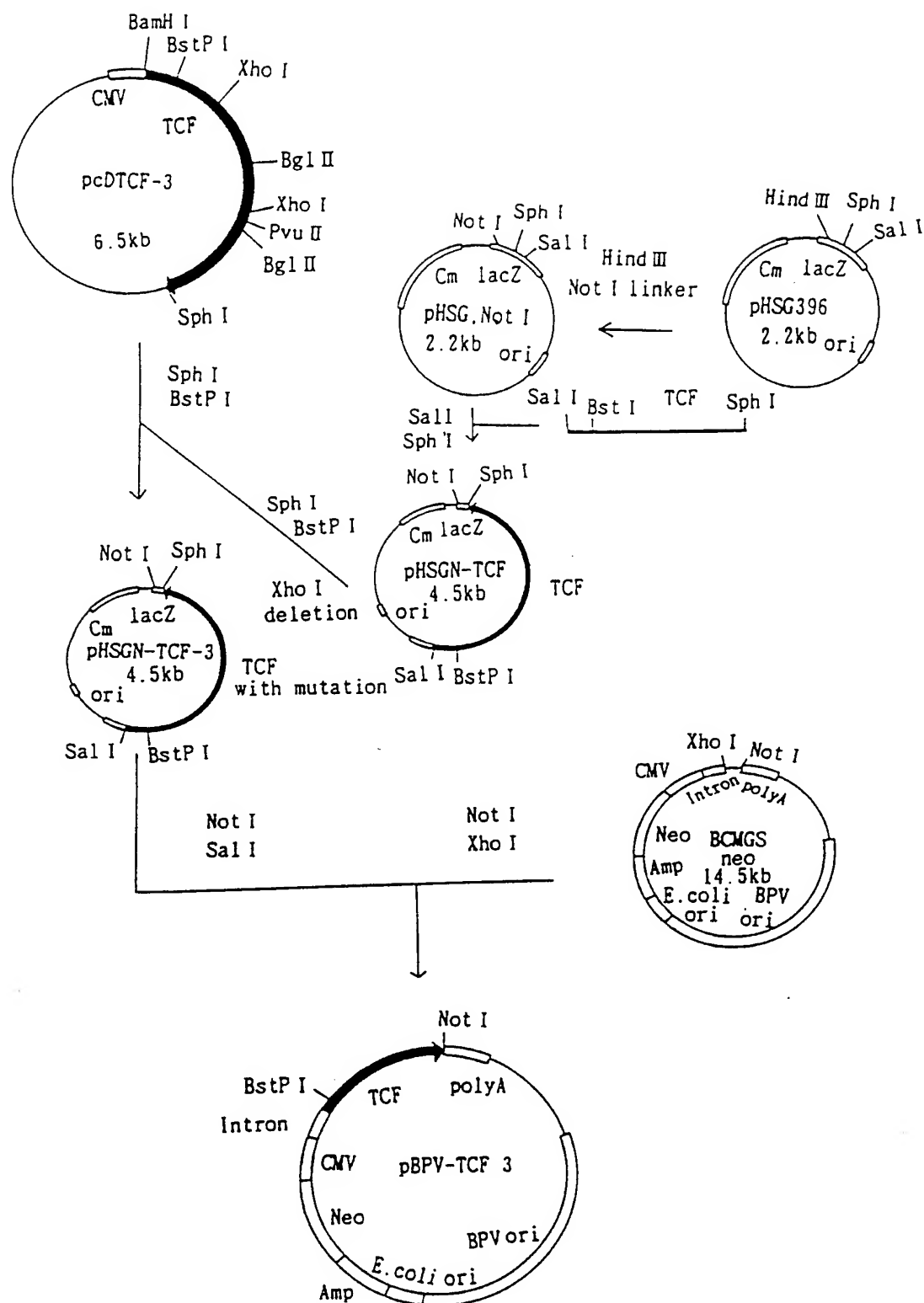


Fig. 7

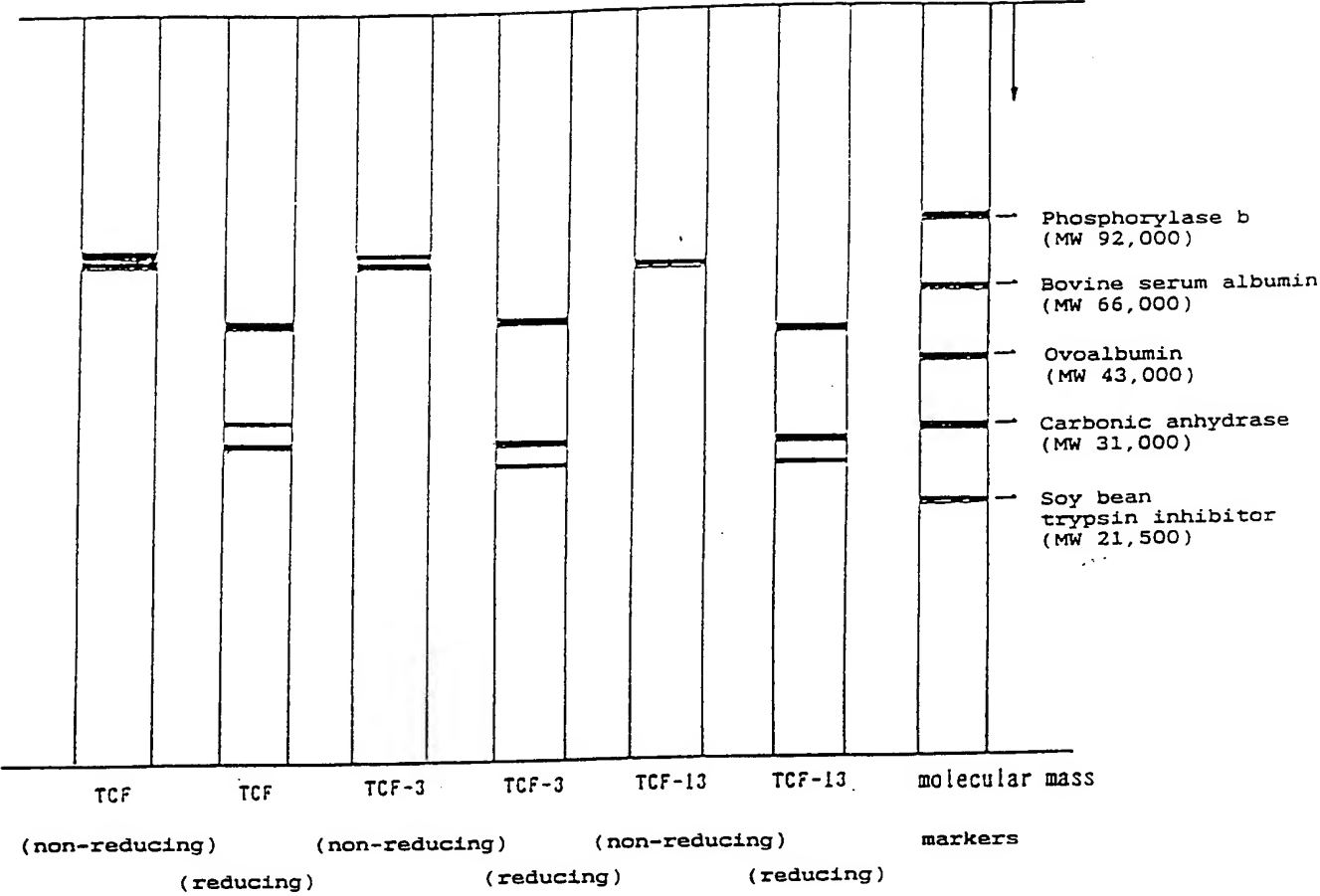
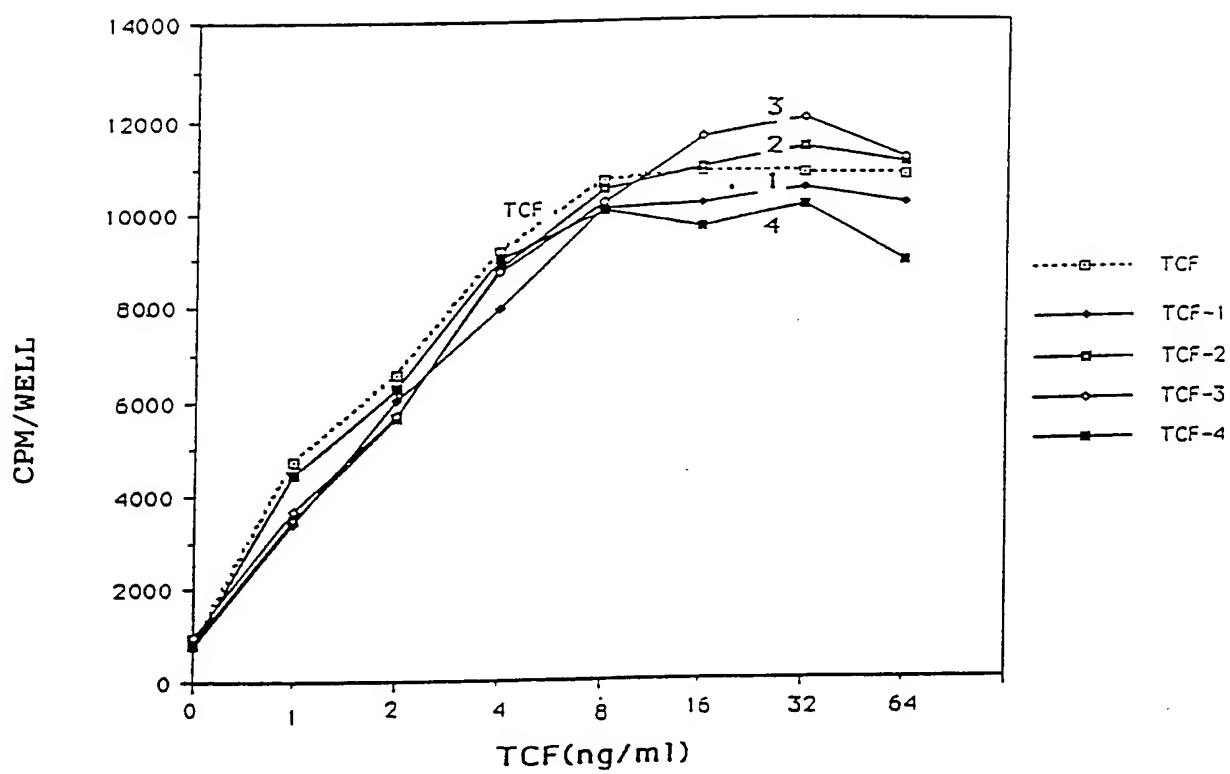
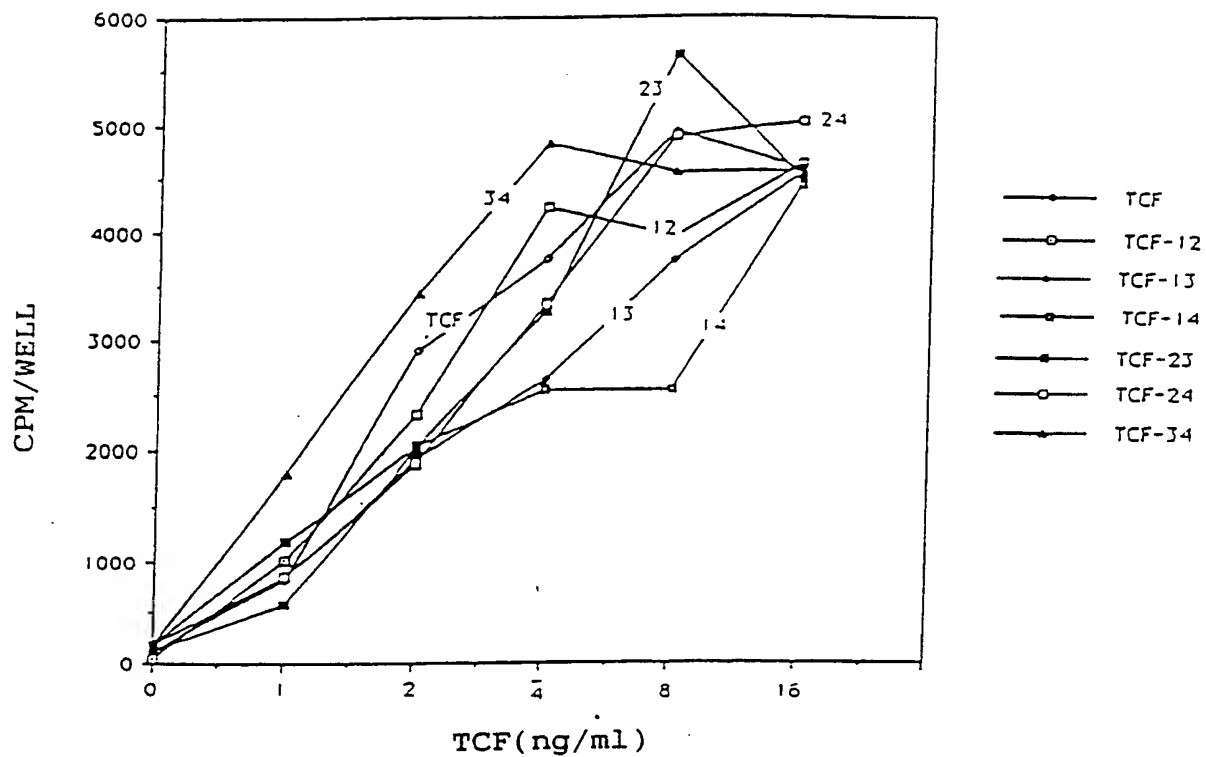


Fig. 8

(1)



(2)



(3)

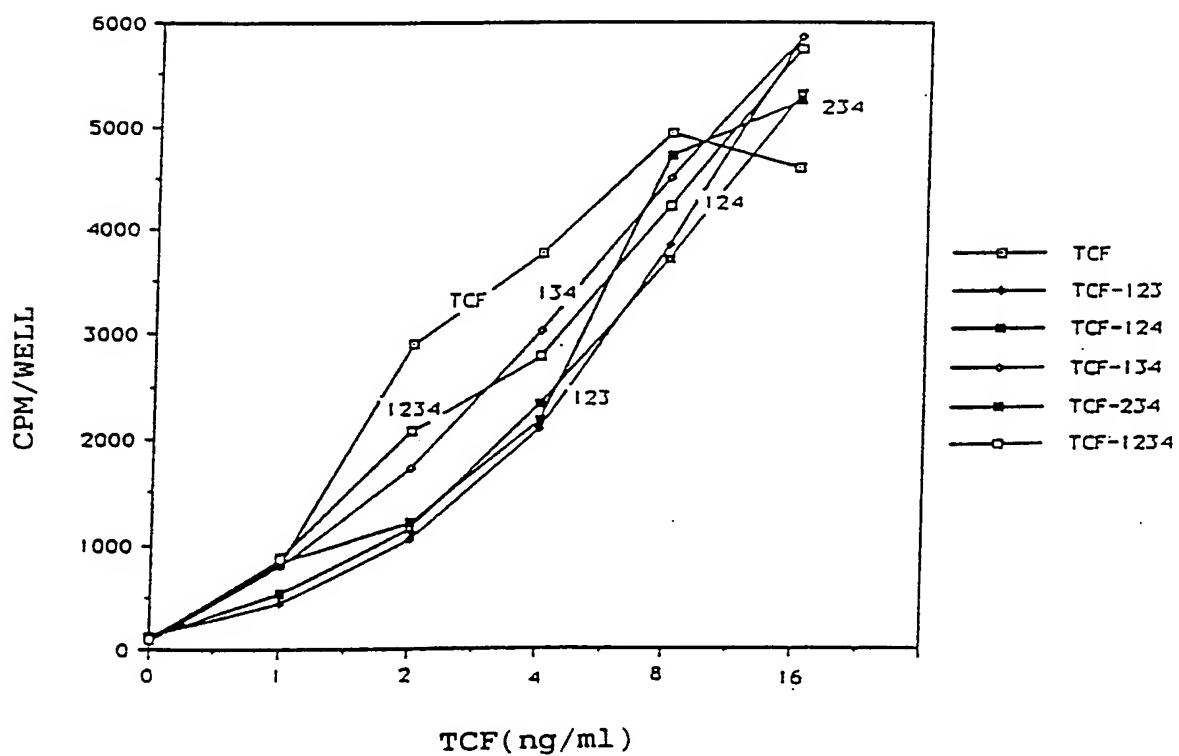


Fig. 9

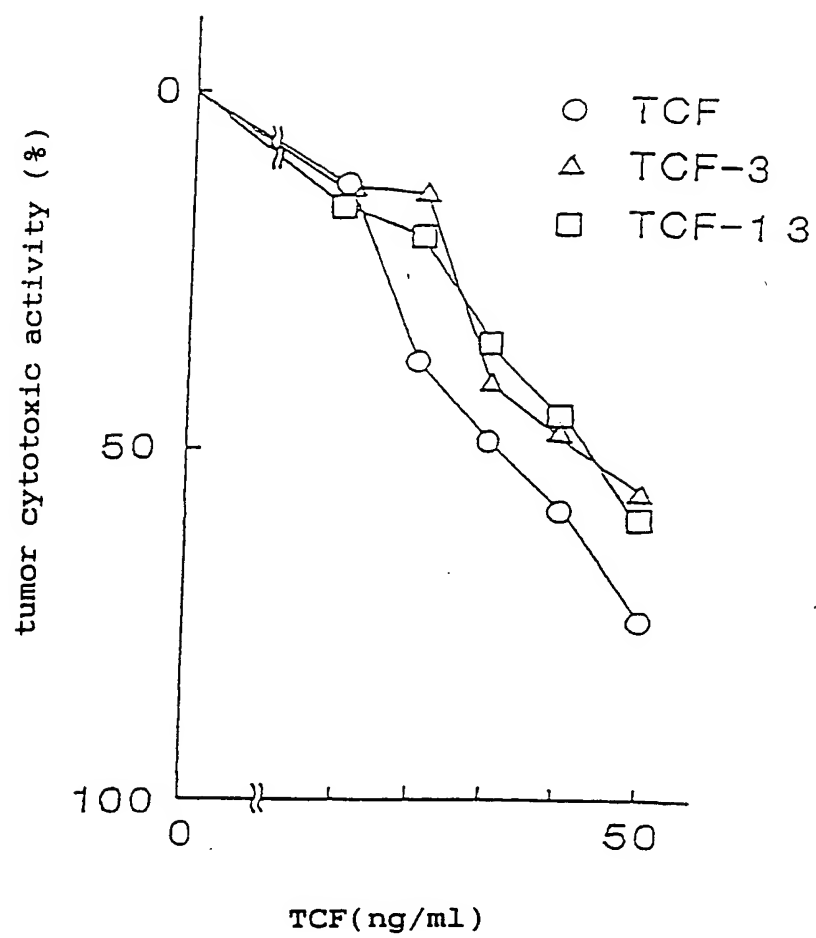
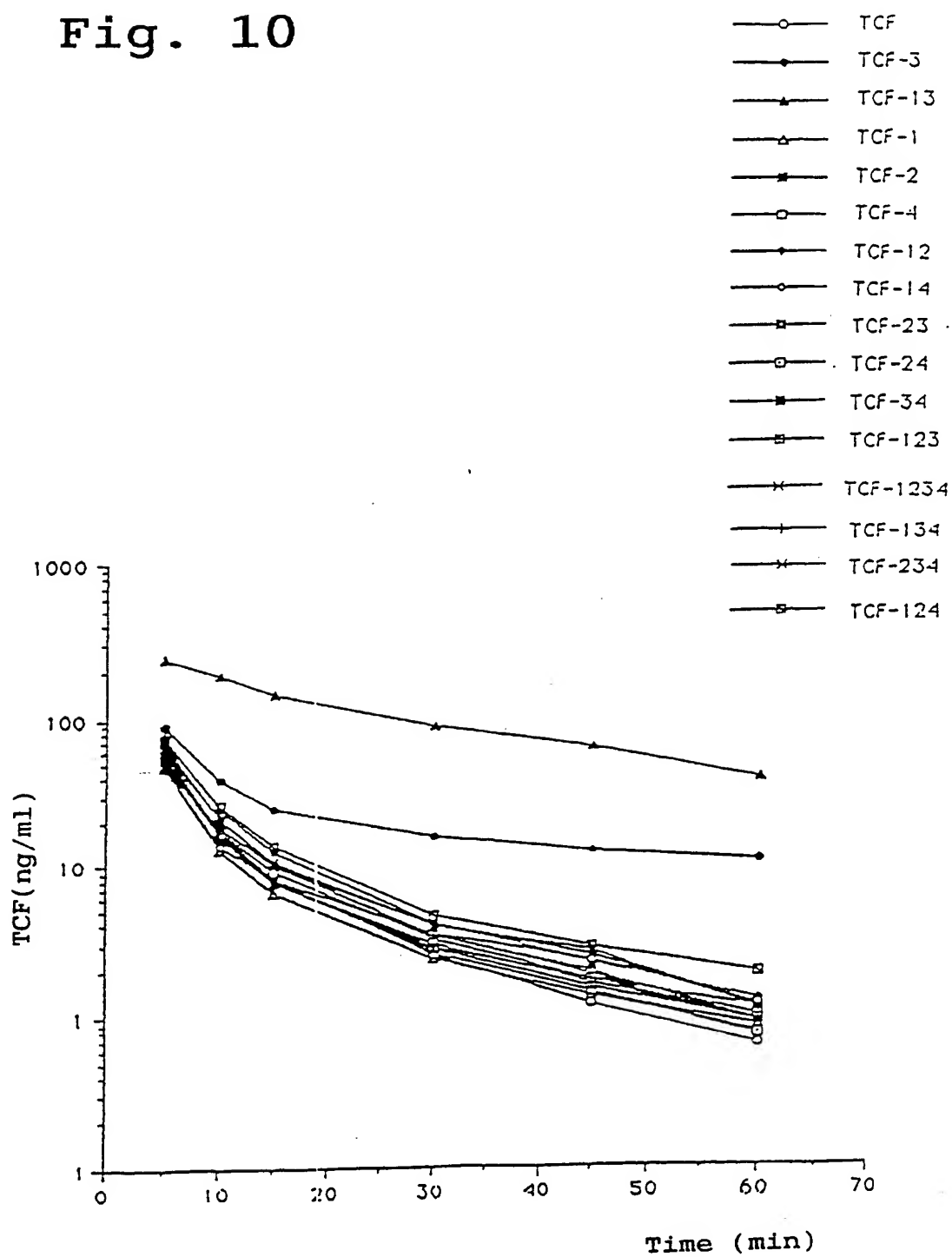


Fig. 10



INTERNATIONAL FORM

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES
OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page.

TO DEPOSITOR:

Name: Research Institute of Life Science
Snow Brand Milk Products Co., Ltd.
General Manager: Gosei KAWANISHI

Address: 519, Aza Hanabayashi, Ohaza Shimoishibashi,
Ishibashi-Cho, Shimotsuga-gun, Tochigi-ken,
329-05, JAPAN

I. IDENTIFICATION OF MICROORGANISM	
Identification Reference Given by the Depositor: pCTCF(S)/MC1061/P3	Accession Number: Bikoken joki No. 3479 (FERM BP-3479)
II. A SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC POSITION	
The microorganism identified under I above was accompanied by a document stating the following item(s). <input type="checkbox"/> A Scientific Property <input checked="" type="checkbox"/> Taxonomic position	
III. RECEIPT AND ACCEPTANCE	
This authority accepts the microorganism identified under I above, which was received on July 13, 1990 (transferred from Bikoken No. P-11605, which was deposited on July 13, 1990)	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Fermentation Research Institute, Agency of Industrial Science and Technology. Ministry of International Trade and Industry Representative: <u>Tomoo SUZUKI</u> (Sealed) Dr., DIRECTOR GENERAL Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, JAPAN Date: July 10, 1991	

INTERNATIONAL FORM

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION
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PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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DEPOSITARY AUTHORITY identified at the bottom
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TO DEPOSITOR:

Name: Research Institute of Life Science
Snow Brand Milk Products Co., Ltd.
General Manager: Yasuyoshi TAKESHITA
Address: 519 Aza Hanabayashi, Ohaza Shimoishibashi,
Ishibashi-Cho, Shimotsuga-gun, Tochigi-ken,
329-05, JAPAN

I. IDENTIFICATION OF MICROORGANISM	
Identification Reference Given by the Depositor: pBPV-TCF-3	Acession Number: FERM BP-4454
II. A SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC POSITION	
The microorganism identified under I above was accompanied by a document stating the following item(s). <input type="checkbox"/> A Scientific Property <input checked="" type="checkbox"/> Taxonomic position	
III. RECEIPT AND ACCEPTANCE	
This authority accepts the microorganism identified under I above, which was received on December 25, 1992	
IV. RECEIPT OF TRANSFER	
This authority received the microorganism identified under I above on December 25, 1992 and transfer of the deposit based on the Budapest Treaty on October 27, 1993 (transferred from Bikoken No. P-13359, which was deposited on December 25, 1992)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Natinal Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology Ministry of International Trade and Industry Representative: <u>Osamu SUZUKI</u> (Sealed) Dr., DIRECTOR GENERAL Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, JAPAN Date: October 27, 1993	

INTERNATIONAL FORM

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION
OF THE DEPOSIT OF MICROORGANISMS FOR THE
PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL
DEPOSITARY AUTHORITY identified at the bottom
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TO DEPOSITOR:

Name: Research Institute of Life Science
Snow Brand Milk Products Co., Ltd.
General Manager: Yasuyoshi TAKESHITA
Address: 519 Aza Hanabayashi, Ohaza Shimoishibashi,
Ishibashi-Cho, Shimotsuga-gun, Tochigi-ken,
329-05, JAPAN

I. IDENTIFICATION OF MICROORGANISM	
Identification Reference Given by the Depositor: pBPV-TCF-13	Acession Number: FERM BP-4455
II. A SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC POSITION	
<p>The microorganism identified under I above was accompanied by a document stating the following item(s).</p> <p><input type="checkbox"/> A Scientific Property <input checked="" type="checkbox"/> Taxonomic position</p>	
III. RECEIPT AND ACCEPTANCE	
This authority accepts the microorganism identified under I above, which was received on December 25, 1992	
IV. RECEIPT OF TRANSFER	
<p>This authority received the microorganism identified under I above on December 25, 1992 and transfer of the deposit based on the Budapest Treaty on October 27, 1993 (transferred from Bikoken No. P-13360, which was deposited on December 25, 1992)</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
<p>Name: Natinal Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology Ministry of International Trade and Industry</p> <p>Representative: <u>Osamu SUZUKI</u> (Sealed) Dr., DIRECTOR GENERAL</p> <p>Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, JAPAN</p> <p style="text-align: right;">Date: October 27, 1993</p>	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/JP 93/01905

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K15/00 C12N1/21 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEM. BIOPHYS. RES. COMM. vol. 170, no. 1, 16 July 1990, US pages 397 - 404 KANJI HIGASHO ET AL. 'Identity of tumor cytotoxic factor from human fibroblasts and hepatocyte growth factor' see "Discussion" ---	1-6
A	BIOCHEM. BIOPHYS. RES. COMM. vol. 180, no. 2, 31 October 1991, US NOBUYUKI SHIMA ET AL. 'Tumor cytotoxic factor...' see "Summary" ---	1-6
A	WO,A,90 10651 (SNOW BRAND MILK CO.) 20 September 1990 cited in the application see Fig. 15 -----	1-6



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

20 April 1994

Date of mailing of the international search report

25-05-1994

Name and mailing address of the ISA

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Authorized officer

Germinario, C

Information on patent family members

PC I/JP 93/01905

Form PCT/ISA/210 (patent family annex) (July 1992)